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Generality of the Branched Pathway in Transcription Initiation by *Escherichia coli* RNA Polymerase*

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Transcription initiation has been assumed to be a multi-step sequential process, although additional steps could exist. Initiation from the T7A1 promoter, in particular, apparently behaves *in vitro* in a manner that can be fully explained by the sequential pathway. However, initiation from the λP_RAL promoter has been shown to follow a branched pathway from which a part of the enzyme-promoter complex is arrested at the promoter raising the question as to which mechanism is general. We found that a moribund complex, characteristic of the arrested branch, is formed at the T7A1 promoter, especially in low salt condition indicating that the initiation mechanism for this promoter is also branched. The results of DNA footprinting suggested that holoenzyme in the moribund complex is dislocated on DNA from the position of productive complex. However, only a small fraction of the binary complex becomes arrested at this promoter, and the interconversion between subspecies of binary complex is apparently more reversible than at the λP_RAL promoter, which explains why the reaction pathway appears to be sequential. These findings suggest a generality of the branched pathway mechanism, which would resolve contradictory observations that have been reported for various promoters.

Transcription initiation in prokaryotes includes at least four events: 1) the binding of holoenzyme to a promoter; 2) the isomerization of the resulting complex accompanied by strand opening; 3) the iterative synthesis and release of abortive transcripts; and 4) the achievement of continuous elongation accompanied by the escape of the enzyme from the promoter (for review see Ref. 1). Although these events are required for transcription initiation in this order, they do not necessarily represent the complete mechanism of transcription initiation, because additional steps could exist. Nevertheless, it has long been assumed that the events listed above is the complete mechanism, mainly because of the lack of evidence for further complications. This simplest mechanism may be called the sequential pathway (Scheme 1*A*). *In vitro*, the initiation from the bacteriophage T7A1 promoter in particular shows the following two behaviors that are characteristic of the sequential mechanism. Firstly, the promoter-RNA polymerase complex synthesizes a stoichiometric amount of full-length transcript in a single-round transcription (2). Secondly, abortive synthesis does not occur after the synthesis of full-length transcript (3), which is consistent with the view that the transcription complex engaged in abortive synthesis is a precursor of the complex synthesizing a long RNA. However, these results do not prove that the sequential pathway is applicable to initiation at all promoters or even at the T7A1 promoter in all conditions.

The above two behaviors characteristic of the sequential pathway are not observed in single-round transcription from the λP_RAL or LacUV5 promoter. The amount of full-length transcript is much less than stoichiometric with the amount of the binary complex (4), and abortive synthesis at these promoters continues long after the completion of full-length transcription, namely persistent abortive synthesis (5), suggesting the existence of an initiation mechanism other than the sequential pathway. These discrepancies are not attributable to heterogeneity in the preparation of the RNA polymerase used for the following reasons. At the λP_RAL promoter, RNA polymerase, which has been isolated from the run-off elongation complex and re-used, displays the same degree of abortive synthesis as the original enzyme, indicating that a fraction of the previously productive enzyme becomes nonproductive (5). Furthermore, the amount of full-length product in single-round transcription from the λP_RAL promoter increases to the same level of the binary complex if GreA, GreB, and a high concentration of the initiation nucleotide are present (6). This observation indicates that nonproductive enzyme can be converted into a productive one. These observations imply the existence of nonproductive pathway(s) that cause(s) the persistent abortive synthesis and that the mechanism of initiation is branched at some stage before abortive synthesis at these promoters. The most plausible model for initiation from the λP_RAL promoter is shown in Scheme 1*B* (5). The moribund complex, which is defined as the ability to synthesize only abortive transcripts, is first generated in the nonproductive branch of initiation, the promoter-arrested pathway. At the λP_RAL promoter, the moribund complex is slowly converted into inactive dead-end complex with a time constant of 10 min in the standard condition (7). Because the moribund complex decays slower than the productive complex, persistent abortive synthesis is observed. The moribund complex is also identifiable in initiation at the *malT* promoter (8). This finding indicates that the branched pathway may arise in initiation at many promoters.

In a test of the generality of the branched pathway, one of the key criteria would be whether or not the initiation at the T7A1 promoter, whose mechanism appears the most sequential, actually follows a branched pathway. Notably, a branched pathway would appear to be sequential if the moribund complex converted into a productive complex before converting into a dead-end complex so that most transcription complexes would finally indulge in productive elongation. In this case, a nearly stoichiometric amount of the full-length transcript should be

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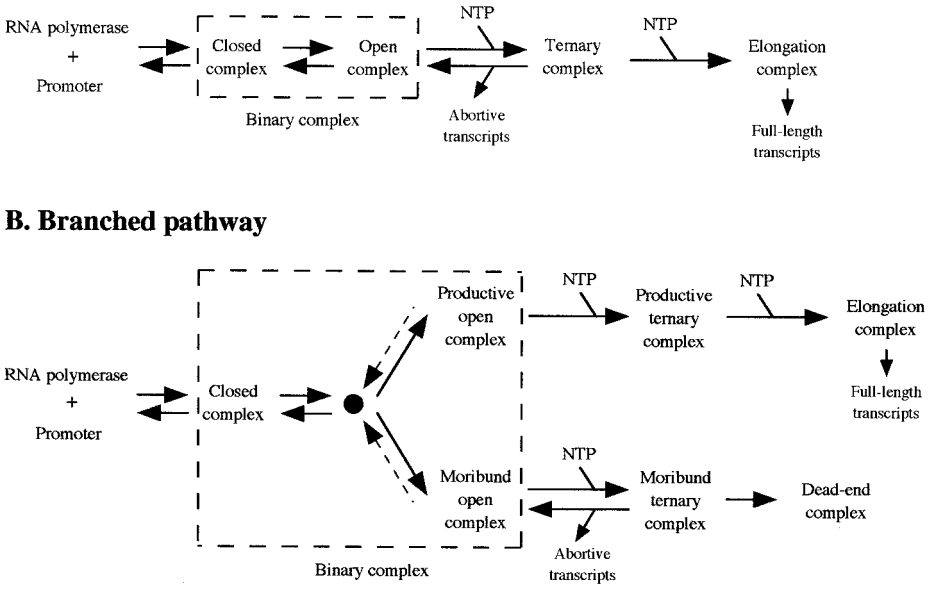

SCHEME 1. **Two models of the initiation pathway for transcription.** *A,* the sequential pathway composed of four essential steps. *B,* the branched pathway that has been applied to the initiation at the λP_RAL promoter. The absence of abortive synthesis in the productive branch has not been established. The *black circle* merely indicates that a branching point exists at the level of the stage of binary complex (within the *broken rectangle*) and could start at one of the subspecies of indicated binary complex. The initiation at the T7A1 promoter is considered to follow this model but with a rapid conversion of moribund subspecies into productive subspecies (*broken arrows*). See the Introduction for details.

synthesized in single-round transcription and production of abortive transcripts should cease before the full-length synthesis is completed. Indeed, this situation occurs at the λP_RAL promoter in the presence of the Gre factors and a high concentration of initiating nucleotide (6).

Here we report that the promoter-arrested pathway can be detected in initiation at the T7A1 promoter. We consistently observe that the interconversion among subspecies of binary complex at the T7A1 promoter is simply more reversible than at the λP_RAL promoter. These findings suggest a generality of the branched pathway mechanism that can explain the seemingly contradictory characteristics of various promoters.

EXPERIMENTAL PROCEDURES

The T7A1 template DNAs used in this study were prepared by PCR using the plasmid pAR1435, which is a derivative of pBR322 with a 102-bp segment of T7 DNA containing the A1 promoter inserted at its *Bam*HI site (9). The other templates were described previously by Sen *et al.* (3). All the transcription assays were carried out as described previously (4, 5) in the standard condition (50 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 0.1 M KCl) or the low salt condition (20 mM Tris-HCl, pH 7.9, 7 mM $MgCl_2$). The dissociation of the binary complex was measured by trapping the dissociated enzyme with a 60-fold molar excess of the 190-bp DNA fragment containing the λP_RAL promoter or with 40 μg/ml heparin. In the electrophoretic mobility shift assay, the binary complex was formed at 37 °C for 10 min in the standard or low salt condition in the presence of 13% glycerol. If necessary, transcription was started by adding the substrate mixture containing 5 μM ATP, 100 μM GTP and CTP each UTP, 0.1 mg/ml heparin and then incubated for 20 min. The digestion with *Hae*III was carried out by incubation with 3.5 units of the enzyme for an additional 30 min before electrophoresis in a 5% polyacrylamide gel in 45 mM Tris borate, pH 8.0, buffer containing 1 mM EDTA. Exonuclease III-mediated DNA footprinting was performed as described previously (7). In Fe^{2+}-induced site-specific radical cleavage, 1.2 pmol of immobilized DNA template harboring the T7A1 or λP_RAL promoter (10) and 1.0 pmol of RNA polymerase were incubated at 37 °C for 10 min in the standard or low salt condition for T7A1 and in the standard or standard +100 mM NaCl condition for λP_RAL. The immobilized binary complex was washed with buffer lacking $MgCl_2$. A final concentration of 0.1 mM $Fe(NH_4)_2(SO_4)_2$ was then added, and the mixture was incubated for an additional 30 min. Cleavage was terminated by the addition of phenol, and the reaction mixture was analyzed in an 8% sequencing gel.

RESULTS

Persistent Abortive Synthesis at the T7A1 Promoter—One important line of evidence for the branched pathway at the λP_R promoter is the occurrence of persistent abortive synthesis. Because this phenomenon disappears in a high salt condition (3), lower salt conditions might favor the formation of the moribund complex at the T7A1 promoter. Although little sign of persistent abortive synthesis at the T7A1 promoter has been detected in the standard salt condition (3), we decided to test for its occurrence at low salt. Transcription from the T7A1 promoter was carried out using a linear 154-bp DNA (Fig. 1*A, template I*) and produced full-length transcripts (74 bases or longer) together with abortive transcripts (shorter than 14 bases) in the standard salt condition (Fig. 1*B*). In the low salt condition, similar amounts of long transcript were synthesized, whereas the average length of abortive products increased slightly. These moderate changes show that no anomalous reactions take place at low salt.

The most sensitive method for the detection of persistent abortive synthesis is a pulse-labeling assay (5) in which transcription is started with unlabeled nucleotides and then the labeled initiating nucleotide [γ-^{32}P]ATP is added at various time points. In this assay, the amounts of the labeled full-length and abortive transcripts indicate the residual proportion of binary complex that can produce the corresponding transcripts at each time point. In the sequential pathway, abortive synthesis should precede the full-length synthesis, and thus the ratio of abortive to full-length transcripts should not increase with the time elapsed prior to addition of labeled ATP. In contrast, this ratio could increase in the branched pathway if the moribund complex has a longer lifetime than the productive one. The observed ratios for the abortive 4-mer or 10-mer transcripts were plotted in Fig. 1, *C* and *D*, against the time of addition of [γ-^{32}P]ATP. In the standard salt condition, the ratios increased by a maximum 4-fold for 60 s, and at low salt they showed 5–20-fold increases. These results indicate that persistent abortive synthesis occurs during initiation at the T7A1 promoter and that deviation from the sequential mechanism is more extensive in the low salt condition.

Arrest of RNA Polymerase at the T7A1 Promoter—Pulse-labeling provides a catalytic assay and thus cannot determine what fraction of binary complex becomes promoter-arrested. Therefore, this fraction was measured by using an electrophoretic mobility shift assay. Promoter-arrested complexes were formed on the 250-bp T7A1 DNA fragment that harbors a

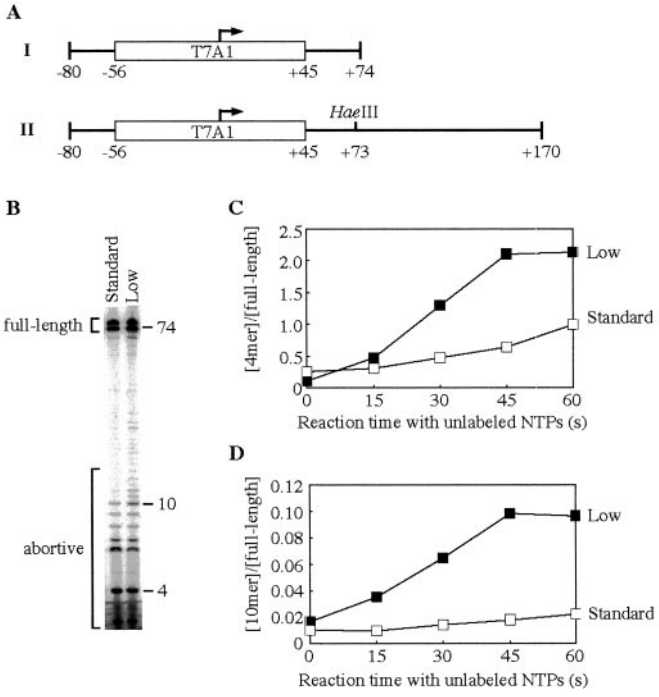

FIG. 1. **Pulse-labeling experiments in the standard and low salt conditions.** *A,* the T7A1 template DNAs used in this study. The *arrows* indicate transcription start sites. The *boxes* indicate the region derived from T7 DNA containing the A1 promoter and the transcription start site. Template I was used in all assays except for the mobility shift experiments in which template II was used. *B,* transcripts synthesized on template I in single-round reactions with 30 nM DNA and RNA polymerase in the standard or the low salt condition as indicated. The *numbers* indicate the lengths of some transcripts. *C* and *D,* the ratios of the amounts of 4-mer (*C*) and 10-mer (*D*) to that of full-length product plotted against the time of addition of [γ-^{32}P]ATP. The *filled* and *open squares* represent the ratios in the low and standard salt conditions, respectively.

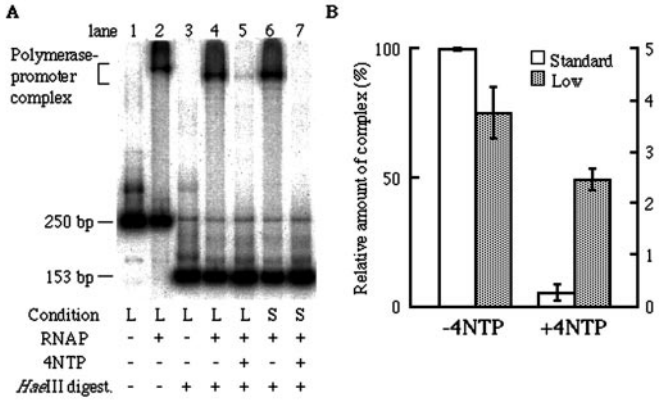

FIG. 2. **Detection of promoter-arrested complexes by gel shift assay.** *A,* the 250-bp fragment (10 nM) with ^{32}P-labeled nontemplate strand was incubated with 25 nM RNA polymerase, and the indicated reactions were carried out. The products were electrophoresed in 5% polyacrylamide gel and visualized by autoradiography. *B,* the amounts of RNA polymerase-promoter complexes observed as shifted band (*153 bp*) + RNA polymerase (*RNAP*) were quantified. Relative amounts of the complexes in the standard (*white bars*) and low (*gray bars*) salt conditions are indicated. The *left* and *right scales* refer to the complexes before and after transcription, respectively. The amount of binary complex in the standard condition is taken as 100%.

*Hae*III site at position +73 (Fig. 1*A, template II*). To distinguish between promoter-arrested and run-off elongation complexes, we removed the unlabeled downstream region of the fragment, which would carry any of the latter complexes, by digestion with *Hae*III. The binary complex was formed in both salt conditions (Fig. 2*A, lanes 4* and *6*). The observed amount of binary complex formed decreased slightly in the low salt condition (Fig. 2*B, −4NTP*) despite the fact that DNA-protein complexes are generally stabilized at low salt. On the other hand, the promoter-arrested complex was almost undetectable under the standard condition (Fig. 2*A, lane 7*) but clearly existed in low salt (*lane 5*), indicating that 2–3% of the binary complex was arrested (Fig. 2*B, right panel*). These results suggest that the fraction of the holoenzyme arrested at the T7A1 promoter is small and that arrest is enhanced at the low salt condition. Therefore, the observed persistent abortive synthesis is a sign of the formation of moribund complex amplified by turnover of this complex. This result explains why nearly stoichiometric amounts of full-length product are obtained at the T7A1 promoter despite the occurrence of arrest at this promoter.

An alternative method to detect the arrested complex is DNA footprinting using exonuclease III (7), which can detect the limits of the region of DNA protected by a bound protein. Thus, we examined the footprints of polymerase bound to the DNA harboring the T7A1 promoter in both conditions. The DNA was footprinted in the absence of holoenzyme or after the formation of the binary complex or 20 min after the addition of 4NTP (Fig. 3). Heparin was added with 4NTP to ensure single-round transcription. No difference between the footprints at the upstream boundary was observed between the two conditions (data not shown). In the standard condition, the footprint on the nontemplate strand of the naked DNA (*lane 2*) was the same as that after transcription (*lane 4*). This finding indicates that little RNA polymerase remains at the T7A1 promoter after 20 min, in agreement with the results of the mobility shift assay. However, in the low salt condition, the footprint of the downstream boundary of the binary complex appears as enhanced bands at positions +9 and +18 to +22 as well as reduced bands at +11 and +13 (*lane 7*). It should be noted that there is a similar footprint after the 20-min transcription in the low salt condition, although the bands are relatively faint (*lane 8*). This observation indicates that a small fraction of the enzyme is still sitting at the promoter, a conclusion again consistent with the finding from the mobility shift assay.

There is a difference between the footprints of the binary complexes in the two conditions. In low salt, the downstream edge of the enzyme footprint is at position +22, whereas in the standard salt condition, it is at +19 (*lanes 3* and *7*). This difference suggests that the RNA polymerase in the binary complex may be more forward-tracked in the low salt condition than in the standard condition. Alternatively, exonuclease III may push or partially displace holoenzyme further upstream in the latter case so that the observed differences reflect not the true boundaries but rather the elasticity of the downstream edge. Whichever is the case, it is clear that there is a physical difference between the downstream boundaries in the two conditions. Here we will tentatively call the change forward tracking. This possibility could result from a structural difference between moribund and productive binary complexes if the earliest branching point occurs at the stage of binary complex formation as in the case of the λP_RAL promoter (6).

Mapping of the RNA Polymerase Catalytic Center onto DNA—One of the most important functional features of the enzyme complex is the location of the catalytic center relative to DNA. Therefore, we examined whether this location shifts in accord with the putative forward tracking of RNA polymerase that was detected by exonuclease III footprinting. According to Zaychikov *et al.* (11), a ferrous ion (Fe^{2+}) is allowed to replace the Mg^{2+} that normally makes a chelate with the catalytic center. The resultant Fe^{2+} chelate generates hydroxyl radicals that cleave the template strand DNA nearby, allowing fine mapping of the catalytic center onto the DNA in the two con-

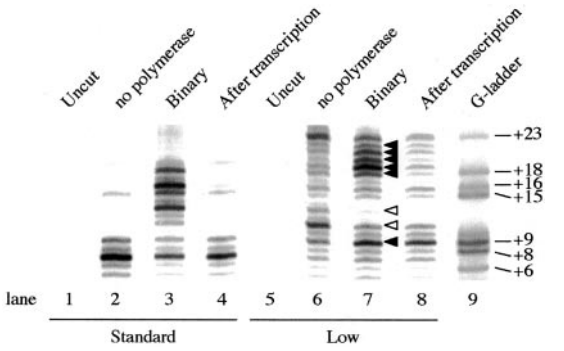

FIG. 3. **Detection of promoter-arrested complexes by exonuclease III-DNA footprinting.** The 5′ end of the nontemplate strand of the DNA harboring the T7A1 promoter was labeled with ^{32}P and footprinted after the reactions indicated. Positions on DNA are indicated relative to the transcription start as +1. Digestion of the DNA was carried out in the absence (*lanes 2* and *6*) or presence (*lanes 3* and *7*) of RNA polymerase or after completion of transcription for 20 min (*lanes 4* and *8*). The reactions were performed in the standard (*lanes 1–4*) or the low (*lanes 5–8*) salt condition. The *open* and *filled triangles*, respectively, indicate the bands with decreased or increased intensities upon the formation of binary complex in the low salt condition.

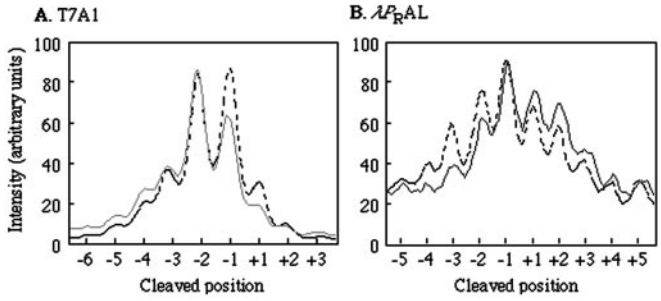

FIG. 4. **The radical cleavage of promoter DNAs induced by Fe^{2+} chelated at the catalytic center of RNA polymerase.** The profiles of band intensities of the template strand are shown for the T7A1 promoter (*A*) and the λP_RAL promoter (*B*). The intensities have been normalized to give the same total density of cleaved bands in both salt conditions. The *broken lines* and the *gray lines*, respectively, indicate cleavage in the low and standard salt conditions. Cleaved positions are indicated relative to the transcription start site as +1.

ditions. Fig. 4 shows the tracings of the autoradiograms obtained by this mapping. The position of the catalytic center of the RNA polymerase-T7A1 promoter complex in the standard condition maps mainly at the position −2 with a secondary peak at −1 (Fig. 4*A, gray line*). In contrast, the mapping in the low salt condition indicates significant forward shifting, such that the two major peaks at −2 and −1 have similar strengths (*black line*). The direction of the movement detected by the Fe^{2+} cleavage is the same as was suggested by the exonuclease III footprinting. Therefore, the binary complex with the T7A1 promoter involves a subspecies that is positioned further forward in the low salt condition. Because the productive subspecies should maintain the same position of the catalytic center relative to the template strand, the forward-tracked subspecies increasing at low salt presumably represents the moribund complex.

This forward tracking of RNA polymerase at the T7A1 promoter in low salt is in sharp contrast to that at the λP_RAL promoter, where back-tracking of the binary complexes as a response to reduced salt concentration is observed (Fig. 4*B*). Thus, at the λP_RAL promoter, the direction of the putative shift of moribund binary complex is the same as that of the dead-end complex (7). Because the moribund complex is functionally defined as a transcription complex that synthesizes only abortive products, its structure could depend on the specific promoter involved, giving a shift in either direction. The relative shift of the catalytic center from the optimal position may decrease the catalytic activity of the subspecies and thus explains why the moribund complex has a lower affinity for the initiation nucleotide and a smaller elongating activity (5).

Confirmation of Rapid Exchange among Subspecies of Binary Complex Formed at the T7A1 Promoter—We have presented two lines of evidence that initiation at the T7A1 promoter follows a branched pathway. The kinetic evidence is the existence of persistent abortive synthesis shown by the pulse-labeling assay, and the biochemical evidence is the existence of arrested complexes detected by mobility shift and exonuclease III-footprinting assays. In both cases, the deviation from the sequential pathway is more distinct in the low salt condition, whereas the pathway in the standard condition appears to be almost sequential. Despite the existence of the moribund complex, almost no dead-end complex is formed in the standard salt condition. This result means that the moribund complex is converted into a productive complex more rapidly than it is inactivated to form a dead-end complex. Therefore, in the standard salt condition, the productive and moribund subspecies formed at the T7A1 promoter are expected to exchange rapidly, whereas those formed at the λP_RAL promoter exchange scarcely.

It is difficult to measure the conversion rates directly in order to confirm this prediction. However, it is possible to measure the overall rate of dissociation from promoters that involves a combination of the exchange reaction and the breakdown of binary complex. Therefore, in the standard salt condition, the overall rate at the T7A1 promoter is expected to be more rapid than that at the λP_RAL promoter. Consistent with this line of reasoning, the reduction of arrest at the λP_RAL promoter, which is produced either by replacing σ^{70} with its region 3 mutant (3) or by the addition of the Gre factors (6), is accompanied by acceleration of the overall dissociation rate. In the case of the mutant σ, biphasic dissociation kinetics was actually observed, presumably one phase because of exchange and the other phase because of breakdown. In the case of the Gre factors, the dissociation kinetics is almost monophasic, suggesting that one of the steps is too rapid to be measured.

We confirmed the existence of the expected rapid exchange at the T7A1 promoter in the standard salt condition by using the DNA fragment harboring the λP_RAL promoter as a competitor of the test promoter fragment. Holoenzyme was preincubated for 10 min with a 1.5-fold excess of the T7A1 promoter fragment to form binary complex, and then a large excess of the competitor was added. At each time point examined, 3NTP and [γ-^{32}P]ATP were added, and reactions were continued for an additional 20 min. Because the promoter on the competitor DNA encodes a G-start, only the initiation from T7A1 was detected. Fig. 5 shows the amounts of the full-length and abortive (6-mer) transcripts. The kinetics are monophasic for both species, and the rate constants are the same (0.2 min^{-1}). As predicted, this value is much larger than that for initiation at the λP_RAL promoter (0.03 min^{-1} for full-length and 9-mer species (3)). This rapid and monophasic decay at the T7A1 promoter is consistent with the expected rapid interconversion of the subspecies of binary complex. A similar rapid decay at the T7A1 promoter was observed in the low salt condition, indicating that the interconversion is still rapid at low salt (data not shown).

Metzger *et al.* (12) reported that the open complex formed at the T7A1 promoter is rapidly inhibited by preincubation with heparin and interpreted this as the result of rapid dissociation of the open complex supposing a competitive role of heparin. Because an allosteric role of heparin was postulated in a transcription study using whole T7 DNA (13), we examined

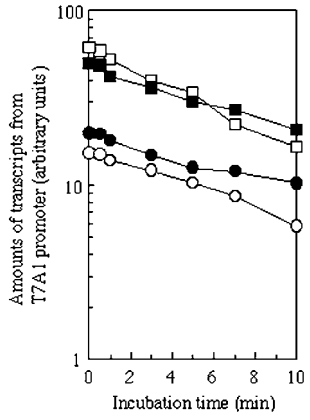

FIG. 5. **Dissociation of the binary complexes at the T7A1 promoter in standard salt condition.** The amounts of the labeled transcripts were plotted against the duration of incubation with the competitors. The *filled squares* and *circles* show the amounts of full-length and 6-mer abortive transcript, respectively, when the λP_RAL DNA was used as competitor. *Open squares* and *circles* show the amounts of the full-length and the abortive transcript, respectively, when heparin was used as competitor.

whether heparin works as a competitor in our experiment. We substituted heparin for the λP_RAL DNA at a concentration sufficient to destroy all of the activity on this template (0.04 mg/ml) when it had been preincubated with holoenzyme. The data for heparin and the λP_RAL fragment agreed within 15%, giving almost the same decay curves (Fig. 5). Therefore, heparin is essentially a competitive inhibitor. This finding is consistent with the observation that for an artificial promoter with an extremely strong affinity for RNA polymerase, a much higher concentration of heparin (5 mg/ml) was needed for the inhibition of transcription with no effects of heparin on transcription at concentrations as high as 1 mg/ml.[1]

DISCUSSION

Among many promoters for *Escherichia coli* RNA polymerase, the A1 promoter of bacteriophage T7 (14) has been one of the best studied *in vitro*, because it has a high affinity for holoenzyme (2, 15) and is among the strongest (16). Using this promoter, the order of binding of substrates (2) and the rate of incorporation of single nucleotides were determined (2, 15) as well as the characteristics of abortive synthesis (17, 18). The concepts of promoter clearance (16) and of initial transcribing complex/initial elongating complex (19) were established by experiments on promoters including T7A1, and the translocational movement of RNA polymerase away from this promoter was systematically studied (20, 21). In addition to these normal features of transcription, elongation arrest was first discovered in the T7A1 transcription unit (22), and hydrolysis of transcripts was also revealed (12, 23). Therefore, the T7A1 promoter is the most representative promoter used in kinetic studies of transcription by *E. coli* RNA polymerase. All of the kinetic results obtained have been interpreted based on the assumption that the mechanism of initiation at this promoter is sequential. However, the new kinetic and structural evidence obtained in this study consistently shows that initiation at the T7A1 promoter occurs by the same branched pathway mechanism, which has recently been established for other promoters such as the λP_RAL (5) and the *E. coli mal*T promoter (8).

This study also shows why initiation at the T7A1 promoter had appeared to be sequential. If the moribund complex converts more rapidly into productive complex than into dead-end complex, the branched pathway becomes almost equivalent to the sequential pathway. The rapid and monophasic dissociation of binary complex observed at the T7A1 promoter suggests that there is indeed a rapid conversion between subspecies of binary complex in standard salt conditions. A similar rapid and monophasic dissociation was observed at the λP_RAL promoter but only in the presence of the Gre factors, which it was concluded introduced reversibility between subspecies of the binary complex (6).

Abortive transcription at the T7A1 promoter was previously investigated by a conventional kinetic assay, which detected persistent abortive synthesis only for a misincorporation product (with A instead of G at the fourth position) at 50 mM NaCl (12). The pulse-labeling assay used in this study is significantly more sensitive and was able to detect persistent synthesis of normal abortive transcripts and did so even in the presence of 0.1 M KCl. Because persistent abortive synthesis has also been observed at the λP_RAL and *lac*UV5 promoters (5), we conclude that there is no qualitative difference between initiation at the T7A1 promoter and others. The difference is only quantitative. The observed common features suggest that the branched pathway mechanism is general among the promoters for *E. coli* σ^{70}-holoenzyme.

In view of the many common features, we are inclined to hypothesize that the earliest branching point of the reaction pathways exists at the stage of binary complex for the T7A1 promoter as already established for the λP_RAL promoter. This hypothesis is substantiated by the results of DNA footprinting with exonuclease III and for Fe^{2+}-induced cleavage at the catalytic center. In the low salt condition, a significant fraction of binary complex is forward-tracked at the T7A1 promoter, and the footprint of the binary complex agrees with that of the promoter-arrested complex obtained after RNA synthesis. This agreement suggests that the forward tracking of the footprint is because of the formation of a significant amount of moribund binary complex. Because the fraction of complex arrested after transcription is only 2–3%, the major fraction of binary complex formed at an early stage is converted into productive complex during RNA synthesis.

The footprints of catalytic center on binary complex dislocated in opposite directions at the T7A1 and λP_RAL promoters when salt concentration was reduced. Although this difference is hard to be explained at present, speculations are possible. One possible hypothesis on binary complex is that the moribund subspecies is too stable in terms of translocation along DNA to escape from a promoter, whereas the productive subspecies is in quasi-stable states that have unfavorable positioning of DNA so that translocation is possible. If the catalytic center locates near the −1 position in the stable state independently of the promoter DNA and if the center in the quasi-stable state is respectively close to −2 or +1 position at the T7A1 or λP_RAL promoter, the observed opposite dislocations are merely a reflection of increased fraction of moribund subspecies induced at low salt. However, other models are equally possible. Irrespective of the models for the moribund complex, the tracking should not be understood as a movement of RNA polymerase molecule but rather as a distortion of the complex, because our footprinting results show that at the T7A1 promoter the upstream boundary of the enzyme does not move irrespective of the movement of downstream boundary.

Mutations in region 3 of σ^{70} are known to increase the ratio of the amount of abortive transcripts to that of the full-length transcript (25). This alteration can be explained by an increase in reversibility between the subspecies of binary complex (3). According to protein footprinting of σ^{70}, this region is protected in holoenzyme as well as in binary complex but exposed in the promoter-arrested complex at the λP_RAL promoter (26), indi-

[1] T. Gaal, R. L. Gourse, and N. Shimamoto, unpublished result.

cating that the structure of this region changes upon the formation of the moribund complex. The region binds to a core enzyme (26, 27) and lies in close proximity to promoter DNA between the −10 and the −35 boxes and at the transcription start site (+1) (28). In fact, these features have been confirmed to exist in the crystal structure of the complex of *Thermus aquaticus* holoenzyme with promoter DNA where the region 3.2 of the σ-subunit binds to the putative exit channel for RNA.[2] Therefore, all of these results including those of the footprinting experiments in this study consistently indicate that structural changes in this part of the binary complex determine the fates of subspecies of the binary complex.

Despite its widespread use in kinetic studies, the T7A1 promoter is not necessarily a typical one in terms of function. The binary complex at this promoter dissociates rapidly in the presence of heparin (12), whereas many other such complexes are insensitive to the reagent. The heparin sensitivity of the binary complex at the T7A1 promoter was shown in the present study to be the result of rapid dissociation of the open binary complex and not to some allosteric effect of heparin peculiar to this complex. The same mechanism of heparin sensitivity was reported for the *rrnB*P1 promoter (24), which is one of the strongest promoters *in vivo* but rather weak under standard test conditions *in vitro*. Therefore, open binary complexes at these promoters equilibrate reversibly with their free components. These findings provide evidence against the widely held mistaken belief that all open complexes are irreversibly formed and resistant to heparin. The reversibility among subspecies of binary complex formed at the T7A1 promoter is intrinsically high as compared with the complexes formed at other promoters that generate dead-end complexes.

Another atypical feature of this promoter is that it directs the synthesis of full-length products far more efficiently than do other promoters. It has been suggested that full-length transcript formation is stoichiometric with the amount of preformed binary complexes at the T7A1 promoter but not at other promoters (4). In other words, the high efficiency is because of conversion of almost all binary complex into the elongation complex, whereas at other promoters only a fraction of binary complex achieve elongation, the rest being excluded. Indeed the nearly full conversion was confirmed for the T7A1 promoter by the results of DNA footprinting and mobility shift assays in this study. In contrast, only a quarter to half of all binary complex clears the λP_RAL promoter (4, 6).

Both the branched and sequential pathways could control the level of transcription in the multiple-round transcription that occurs *in vivo*. However, there are two distinct characteristics that are specific to the branched pathway. The formation of dead-end complex attenuates the level of transcription irrespective of the location of the rate-limiting step in transcription initiation, whereas in the sequential pathway, kinetic control is effective only at the rate-limiting step. Therefore, initiation could be regulated by the branched pathway, even if the rate-limiting step changes according to the physiological environment. The second special characteristic of the branched pathway is that it could amplify the effects of repressors and activators. For example, if a repressor inactivates RNA polymerase-promoter complex in the branched pathway, the inactivation could be maintained long after dissociation of the repressor, and the promoter could remain blocked until the arrest was relieved. Such persistent repression would prevent the formation of a queue of RNA polymerases in a transcription unit, which would be expected if regulation were the result of an arrest in elongation.

[2] K. Murakami and S. A. Darst, unpublished result.

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Generality of the Branched Pathway in Transcription Initiation by *Escherichia coli* RNA Polymerase

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